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Influence of protozoan grazing on contaminant biodegradation

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Abstract

The influence of protozoan grazing on biodegradation rates in samples from contaminated aquifer sediment was evaluated under aerobic and anaerobic conditions. Predator–prey biomass ratios suggested that protozoan grazing might be influencing bacterial populations. Experiments under aerobic conditions were conducted with a sediment extract fed with BTEX and treated with protozoan inhibitors (cycloheximide, neutral red, amphotericin-B). After 10 days, BTEX losses were enhanced in the presence of protozoan inhibitors, suggesting that reduced protozoan grazing enhanced the rate of BTEX biodegradation. In tests conducted in macrocosms under anaerobic conditions, treatments included benzaldehyde (carbon substrate), benzaldehyde+cycloheximide, a live control (no carbon), and an abiotic control. In both the benzaldehyde-only and benzaldehyde+cycloheximide treatments, repeated benzaldehyde additions resulted in an increase in the total fermenter population from 10^3 to 10^5 cells (g sediment)$^{-1}$ and in the Fe-reducing population from 10^1 to 10^5 cells g^{-1}. However, the protozoan population remained at about 20 cells g^{-1} in the sediment with no cycloheximide, and there was no difference in benzaldehyde biodegradation in the presence and absence of cycloheximide, suggesting that predation was not a significant control on anaerobic benzaldehyde biotransformation. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Groundwater aquifers have been contaminated with a wide variety of hazardous organic chemicals as a result of both primitive waste disposal practices and accidental releases [1,2]. Common contaminants include petroleum hydrocarbons, chlorinated solvents, ketones and ethers. With the input of carbon into aquifers, the oxygen in the aquifers is typically depleted and anaerobic conditions often prevail. The anaerobic degradation of organic contaminants has been demonstrated by microbially mediated reaction pathways, including nitrate reduction [3,4], iron reduction [5,6], sulfate reduction [7,8], and methanogenesis [9,10]. Furthermore, microorganisms are known to play a significant role in the regulation of contaminant fate and transport in contaminated aquifers [11].

One strategy for the remediation of contaminated aquifers is natural attenuation, in which the activity of the indigenous microbial population is sufficient to control contaminant migration and ultimately to consume the contaminants. One factor limiting the consideration of natural attenuation in remediation

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programs is uncertainty regarding the rate of contaminant biodegradation. Biodegradation rates calculated from field data vary widely. Reported first-order degradation rates for benzene range from undetectable to approximately 1% per day [12]. For natural attenuation to be considered in the development of remediation programs, a more fundamental understanding of factors controlling biodegradation is needed.

Protozoan grazing is one factor controlling the abundance of bacteria in freshwater and marine environments [13–16] and in wastewater treatment plants [17,18]. Recent studies have shown that protozoa are common in both shallow [19–23] and deep (over 200 m below surface) aquifers [24], and elevated protozoan populations have been detected in contaminated aquifers. This suggests that protozoan populations increase because of grazing on bacterial populations that are stimulated by an influx of carbon [25–27]. Natural attenuation rates in a gasoline-contaminated aquifer at Rocky Point, NC, were lower than at many other sites [12], raising the possibility that protozoan grazing was reducing biotransformation rates in the aquifer.

The objective of this study was to evaluate the influence of protozoan grazing on contaminant biodegradation rates in a gasoline-contaminated aquifer undergoing intrinsic bioremediation in Rocky Point, NC.

2. Materials and methods

2.1. Field site

Sediment and groundwater were obtained from a field site in Rocky Point, NC, where an underground storage tank leaked gasoline to a shallow aquifer. Previous research has shown that benzene, toluene, ethylbenzene and xylene isomers (BTEX) are biodegraded by the indigenous microbial population and that iron reduction is a significant electron acceptor [28,29].

2.2. Experimental design

Initially, sediment samples were analyzed to determine if protozoa were present in the aquifer. Aerobic and anaerobic protozoa and total bacteria (acridine orange direct count) were enumerated in pristine and contaminated sediment samples. Three pristine and six contaminated samples were analyzed for protozoa and total bacteria at intervals of 2–3 cm. Another six contaminated samples were analyzed for protozoa and total bacteria at 30 cm intervals.

Once the presence of protozoa was documented, laboratory experiments were conducted to evaluate the influence of protozoan grazing under both aerobic and anaerobic conditions. The objectives of the first experiment were: (1) to screen a number of protozoan inhibitors under aerobic conditions for identification of a suitable inhibitor for use under anaerobic conditions; and (2) to evaluate whether protozoan grazing could be significant under aerobic conditions. Aerobic conditions were employed because the population of aerobic protozoa exceeded that of anaerobes in the sediment tested and results could be obtained more rapidly than under anaerobic conditions. Laboratory microcosms (20-ml test tubes) were inoculated with an extract of contaminated sediment known to contain protozoa. Three protozoan inhibitors (cycloheximide, neutral red, amphotericin B) were tested in duplicate over a range of concentrations. Inhibitor concentrations were: cycloheximide, 100, 500, 1000 and 2500 mg l^{-1}; neutral red, 10, 50 and 100 mg l^{-1}; and amphotericin B, 10, 50 and 100 mg l^{-1}. Cycloheximide inhibits 80S ribosomal protein synthesis, neutral red stimulates autocystosis and inhibits encocystosis, while amphotericin B is a polyene antifungal agent, which apparently binds to sterols and damages the osmotic barrier of the plasma membrane of eukaryotes [30,31]. The initial substrate concentration was 0.4–0.5 mg l^{-1} of each BTEX component.

Based on the results of the first experiment, the effectiveness of cycloheximide was confirmed in a subsequent experiment. Treatments included triplicate live, abiotic (heat-killed), and live+cycloheximide (100 and 1000 mg l^{-1}) microcosms. The initial substrate concentration was 2 mg l^{-1} of each BTEX component. In both experiments, effects were evaluated based on the extent of BTEX biodegradation over 10 days. All sediment was collected from a contaminated region of the Rocky Point aquifer.

Tests under anaerobic conditions were conducted in 1-l bottles (macrocosms) that contained contami-

nated aquifer sediment. Treatments consisted of benzaldehyde, benzaldehyde+cycloheximide (1000 mg l^{-1}), a live control to which no carbon was added, and an abiotic control. Substrate biodegradation rates and both protozoan and bacterial populations were monitored. Benzaldehyde was selected because it is a rapidly degradable substrate that would amplify the rate and magnitude of population shifts relative to toluene. Its biodegradation in this aquifer sediment was verified in preliminary work. In addition, benzaldehyde is a suspected intermediate in toluene metabolism under iron-reducing conditions [6].

2.3. Sediment and groundwater collection

Sediment cores for population enumeration were collected at two intervals from the contaminated region: 2.13–2.74 and 2.74–3.35 m. Samples from the pristine region were collected from 2.74–3.35 m. All sediment was obtained under anaerobic conditions by drilling below the water table with a hollow stem auger and then by advancing a sterile coring tube. The tube was brought to the surface, immediately capped with sterile butyl rubber stoppers, and transported to the laboratory on ice where it was extruded in an anaerobic glove box (Ray Products, El Monte, CA) under N_2 within 12 h. During extrusion of the sediment cores, three adjacent samples from each core, each 2–3 cm long, were collected at 2.4 m (contaminated) and 3.05 m (pristine and contaminated). Aquifer sediment at 30-cm intervals was collected between 3.05 and 4.88 m in a sterile plastic sleeve (3.8-cm-diameter × 1.2-m-long). In the laboratory, the sleeve was cut open and divided every 0.3 m in an anaerobic glove box. Each sample was mixed separately in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). Inocula from all sediment samples were formed within 36 h of collection.

Sediment for the BTEX (aerobic) and benzaldehyde (anaerobic) biodegradation experiments was obtained from a region approximately halfway between the source area and the end of the plume. Samples for the aerobic and anaerobic experiments were obtained at depths of 2.13–2.74 and 3.51–4.12 m, respectively. In the laboratory, the first and last 10 cm of the core were removed and the outer portions of the core were pared away. Sediment was then transferred into sterile mason jars and stored at 4°C. Samples were mixed in the anaerobic chamber prior to use.

Groundwater was collected anaerobically from the same location as the sediment. The well headspace was sparged with argon for 10 min before and during sample collection. Groundwater was pumped from the well through a closed system of polyethylene tubing equipped with a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI) and collected in a N_2-sparged bottle. The water was transported to the laboratory on ice where it was stored at 4°C. All equipment and containers in contact with the water and sediment were presterilized.

2.4. Microcosm construction

Microcosms were constructed in sterile 20-ml pressure tubes (Belco Biotechnology, Vineland, NJ) that contained 15 ml of a sediment extract. The extract was formed by shaking 100 g of wet sediment in 1 l of filter-sterilized groundwater. The tubes were capped with sterile black butyl rubber stoppers and crimped with an aluminum seal. Abiotic treatments were sterilized by autoclaving (121°C, 1 h). All tubes were spiked with solutions containing the appropriate inhibitor and BTEX, and the tubes were incubated at 25°C.

2.5. Macrocosm construction

Macrocosms were constructed in 1-l bottles containing aquifer sediment (1000 g) and groundwater (600 ml) and were capped with a rubber stopper. Benzaldehyde was repeatedly added to the two live treatments. In the abiotic control, the sediment was autoclaved for 1 h on 2 consecutive days, after which $HgCl_2$ (250 mg l^{-1}) was added. The abiotic (killed control) received benzaldehyde (25 mg l^{-1}) and cycloheximide (1000 mg l^{-1}). All macrocosms were constructed in an anaerobic chamber using aseptic techniques and incubated in the chamber at 25°C. Aqueous samples from the macrocosms were analyzed for benzaldehyde, cycloheximide and $Fe(II)_{aq}$. Duplicate sediment samples for enumeration of anaerobic protozoa, total fermenters and Fe reducers were removed by using a sterile, hollow glass rod.

2.6. Inhibitor preparation

Stock solutions of eukaryote inhibitors were prepared in distilled water at concentrations of 10 g l^{-1} and stored at 4°C. Prior to use, cycloheximide was warmed to 60°C to increase its solubility. Required volumes of the stock solutions were filter-sterilized (0.2 μm) prior to use.

2.7. Microbial population enumeration

Acridine orange direct counts (AODC) were conducted as described previously [32]. The total fermentative, Fe-reducing and aerobic and anaerobic protozoan populations were enumerated using most probable number (MPN) techniques. Inocula were prepared by shaking 10 g of sediment in 90 ml of sterile groundwater. Inocula were then diluted in sterile groundwater for MPN assays. MPNs were calculated by using a computer program [33].

The populations of aerobic and anaerobic protozoa were enumerated by using the five-well MPN procedure [21,25]. The procedure is based on the addition of an inoculum to a glass ring immobilized with agar in a Petri dish. *Enterobacter aerogenes* was grown separately on trypticase soy agar and added to each cylinder as a food source for protozoa. One ml of the inoculum was added to five replicate rings at each dilution. For anaerobic protozoa, Petri dishes were incubated in the anaerobic chamber at 25°C. Wells were examined microscopically for the presence of protozoa and were counted as negative if none were visible after 1 or 2 months for the aerobic and anaerobic MPNs, respectively. Encysted protozoa were enumerated by treating a separate subsample of the sediment extract with 10 ml of 0.55 N HCl for 15 min to kill vegetative protozoa. A greater MPN in the untreated sample relative to the treated sample indicated the presence of vegetative protozoa.

Total fermenters were enumerated by five-tube MPN tests with the following medium (g l^{-1}): glucose (10), yeast extract (1), trypticase peptones (2), KH_2PO_4 (1.61), $Na_2HPO_4 \cdot 7H_2O$ (3.18), NH_4Cl (1), NaCl (0.9), $MgCl_2 \cdot 6H_2O$ (0.2), $CaCl_2 \cdot 2H_2O$ (0.1), $NaHCO_3$ (3.5), and cysteine hydrochloride (0.5). In addition, the medium contained trace minerals [34] with the addition of Na_2WO_4 (0.033) and vitamins [35]. Resazurin (2 ml, 0.1%) was added to the medium and the pH was adjusted to 6.6. The medium was boiled under N_2 that had been passed through a heated copper column to remove traces of oxygen. Nine ml of medium were dispensed in anaerobic pressure tubes, which were then sealed with black butyl rubber stoppers and aluminum crimps and autoclaved (121°C, 20 min). After 1 month, tubes were scored positive based on elevated optical density.

Iron reducers were enumerated by using a 10-tube MPN assay with medium selective for Fe(III) reducers [36]. The medium was sparged with N_2 to remove dissolved oxygen and the pH was adjusted to 7. Finally, 9 ml were dispensed into anaerobic pressure tubes and sealed as above. Tubes were incubated for 2 months, after which $Fe(II)_{aq}$ was measured in each tube. Tubes were considered positive if $Fe(II)_{aq}$ exceeded that in uninoculated controls at the 99% confidence interval. Three uninoculated controls were analyzed at each dilution. For Fe(II) analysis, a 0.5-ml sample was digested in 5 ml of 0.75 N HCl under anaerobic conditions. After 3 h when the liquid was clear, tubes were removed from the anaerobic hood and 5.5 ml of phosphate buffer (112.7 g KH_2PO_4 l^{-1} and 144.9 K_2HPO_4 l^{-1}) containing phenanthroline (1 powder pillow per 25 ml) was added (Hach, Loveland, CO). A_{510} was then read after 15 min and compared to a standard curve.

2.8. Monitoring and analytical methods

Microcosms were analyzed for BTEX by first removing approximately 0.2 ml of free liquid in a gas-tight syringe. The sample was diluted to 5 ml and analyzed for BTEX with a Tekmar Purge-and-Trap Model LSC 2000 and a Perkin Elmer autosystem gas chromatograph (GC) equipped with a 75-m DB-624 megabore capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector. The injector temperature was maintained at 250°C. The oven was initially set at 40°C for 2 min, increased at 20°C per min to a final temperature of 140°C and held for 5 min. The detection limit for BTEX compounds was 1 μg l^{-1}.

To analyze macrocosms for benzaldehyde and cycloheximide, 2 ml of free liquid was removed using a gas-tight syringe while puncturing the stopper with a needle so that a vacuum did not develop. Sampling was conducted in the anaerobic chamber and the

sample volume was replaced with sterile groundwater. One μl of sample was analyzed by direct injection to the GC described above. The injector temperature was maintained at 250°C. The oven was initially at 40°C for 2.5 min, increased at 10°C per min to 75°C and then increased at 20°C per min to a final temperature of 200°C and held for 5 min. The detection limits for benzaldehyde and cycloheximide were 1 and 10 mg l^{-1}, respectively.

3. Results

3.1. Distribution of protozoan populations

Comparison of protozoan populations in closely spaced (2–3 cm) contaminated and pristine samples, as measured by MPN tests, shows elevated populations in the contaminated samples (Table 1). Although MPN techniques have inherent limitations in the fraction of the population measured [37], MPN values were used in this study as a relative measure to evaluate spatial and temporal differences in populations.

One explanation for the increased protozoan populations in the contaminated sediment is that there was an increased bacterial population on which to graze because of the increase in organic carbon. Aerobic protozoan populations in the contaminated samples declined with depth. In addition, it would not appear that this decrease can be attributed to decreased redox potential as the anaerobic protozoan population did not increase between sample sets A and B (Table 1). In addition, the upper sediment would occasionally be exposed to aerobic conditions during periods of high groundwater recharge. The overall decline of protozoan populations with depth is consistent with previous reports [21,38]. In contrast to populations in the closely spaced samples, there was substantial variability in protozoa in samples enumerated at 0.3-m intervals (Table 1).

3.2. Predator–prey relationships

Microbial population data (Table 1) were used to evaluate whether protozoan–bacteria ratios were consistent with the potential for protozoan gazing to be important. Theoretical models of steady-state

Table 1
Distribution of aerobic and anaerobic protozoa and total bacteria (AODC) in contaminated and pristine samples (cells g dry $wt.^{-1}$)

Sample[a]	Aerobic protozoa (MPN)	Anaerobic protozoa (MPN)	Total bacteria (AODC)	Predator–prey biomass ratio[b]
A1	35	4.7	3.6×10^6	0.16
A2	198.4	2.0	2.6×10^6	0.89
A3	193.7	1.9	3.0×10^6	0.87
B1	<0.21	0.7	4.6×10^6	0
B2	0.8	0.8	5.0×10^6	0.06
B3	8.4	1.1	5.3×10^6	0.04
C1	<0.21	0.4	3.0×10^6	0
C2	<0.21	<0.21	8.8×10^5	0
C3	<0.21	0.8	1.4×10^6	0
I	25.2	<0.21	2.3×10^6	0
II	<0.21	2.6	2.2×10^6	0.02
III	<0.21	105.2	2.5×10^6	0.95
IV	<0.21	47.7	2.3×10^6	0.43
V	4.8	2.4	2.5×10^6	0.02
VI	1.4	0.5	2.9×10^6	0

[a]Samples A1–A3 are closely spaced samples (2–3 cm apart) taken at 2.4 m from the contaminated region. Samples B1–B3 are closely spaced samples (2–3 cm apart) taken at 3.05 m from the contaminated region. Samples C1–C3 are closely spaced samples (2–3 cm apart) taken at 3.0 m from the pristine region. Samples I–VI were recovered at 3.05, 3.35, 3.66, 3.97, 4.27 and 4.88 m, respectively, from the contaminated region. The sample recovery distances represent the depth below the ground surface.
[b]Calculation described in Section 3.2.

Table 2
Effect of protozoan inhibitors on BTEX biodegradation

Treatment	Inhibitor concentration (mg l^{-1})	Benzene loss[a]	Toluene loss[a]	*o*-Xylene loss[a]	BTEX loss[a,b]
Control		25.9 (13.4)	46.2 (15.6)	10.8 (6.9)	37.0 (6.0)
Amphotericin B	10	13.0 (4.9)	17.9 (13.3)	3.0 (7.8)	24.0 (0.6)
Amphotericin B	50	35.7 (1.5)	28.5 (7.1)	13.2 (9.9)	34.6 (1.5)
Amphotericin B	100	52.2 (35.6)	51.6 (23.5)	32.9 (26.2)	47.2 (22.4)
Neutral red	10	49.3 (5.2)	57.8 (3.0)	27.0 (4.1)	48.6 (2.5)
Neutral red	50	67.0 (2.8)	64.9 (1.2)	25.8 (3.1)	53.3 (2.8)
Neutral red	100	75.3 (2.2)	66.6 (1.8)	41.0 (1.1)	60.1 (1.3)
Cycloheximide	100	78.2 (0.8)	67.7 (0.1)	58.8 (3.9)	64.9 (1.5)
Cycloheximide	500	77.6 (0.5)	67.0 (1.7)	55.2 (0.9)	63.2 (0.7)
Cycloheximide	1000	78.7 (0)	70.3 (0.5)	59.3 (3.1)	69.7 (0.9)
Cycloheximide	2500	67.9 (1.8)	68.6 (0.3)	34.9 (32.6)	57.2 (7.6)

[a]Data are expressed as the % loss after 10 days after correction for the abiotic loss. Data represent the average of two tests for the treatments and three tests for the controls. The standard deviation is presented parenthetically.
[b]The sum of benzene, toluene, ethylbenzene and *o*- and *m*-xylene.

phagotrophic food chains suggest that there is a characteristic ratio between the biomass of predators and their prey [39,40]. The value of this ratio is derived from the ratios of individual predator and prey sizes and the size-dependent rates of metabolism and growth, making this ratio a function of growth efficiencies. Specifically, the ratio between predator and prey biomass is proportional to the gross growth efficiency of the predator (i.e. yield = assimilated C/consumed C).

Predator–prey biomass ratios were calculated for contaminated and pristine samples (Table 1). Ratios were calculated assuming a dry matter content of 20%, a carbon content of 50% of dry weight and a cell density of 1.05 wet g cm^{-3}. The average total bacterial population was about 2×10^6 cells per gram of sediment. Assuming the biovolume of a bacterium to be 0.2 μm^3 [41], a biovolume to biomass conversion factor of 0.121 pg C μm^{-3} was calculated which results in a total bacterial biomass of 48.4×10^{-6} mg C (g sediment)$^{-1}$. The biovolumes of protozoa were calculated assuming the diameter of amoebae and flagellates to be 20 and 5 μm, respectively, resulting in a calculated biomass for amoebae and flagellates of 0.44 ng C and 0.006 ng C per organism, respectively.

To calculate predator–prey ratios, only aerobic protozoan biomass was taken into account for samples A1–A3 because aerobic protozoa were dominant. Amoebae and flagellates were detected in equal number in these samples. The predator–prey ratios in samples A1–A3 were clearly elevated relative to the pristine samples and samples B1–B3. In samples I to VI, only anaerobic protozoan biomass was taken into account because anaerobes were dominant. Also, only amoebae and no flagellates were present in these samples. The elevated predator–prey ratio in selected contaminated sediment samples suggests the potential importance of protozoa in these samples.

Predator–prey biomass ratios were also calculated for samples from other contaminated aquifers, where reports have suggested that protozoa influence biodegradation, and for samples from pristine aquifers [21,22,25,26,42,43]. The contaminated aquifers had some samples with elevated predator–prey ratios, while the ratios were always low in the samples from pristine aquifers. In an aviation-fuel-contaminated aquifer, the predator–prey ratio was greater than 10 in samples where H_2O_2 stimulated bacterial growth [25].

3.3. Aerobic experiment

The effects of amphotericin B, neutral red and cycloheximide on aerobic BTEX biodegradation are presented in Table 2. Data for ethylbenzene and *m*-xylene are not reported because biodegradation was sufficiently rapid in the ambient treatments that there were no effects attributable to the inhibitors. Generally, biodegradation was enhanced in the presence of amphotericin B at 100 mg l^{-1} and all concentrations of neutral red and cycloheximide. The largest stim-

ulation was observed for *o*-xylene. The most likely explanation for this is that *o*-xylene biodegradation was slowest in the controls and there was more potential for stimulation. The slight decrease in biodegradation at the highest cycloheximide concentration may indicate some inhibition of bacteria. The biodegradation data indicate that when protozoan activity is suppressed, biodegradation is enhanced, presumably due to the removal of one regulator of bacterial growth. Similar observations have been reported for *p*-nitrophenol biodegradation in lake water [44,45].

A confirmatory test was conducted with cycloheximide because it was effective at the lowest concentration tested (100 mg l^{-1}) and was not inhibitory at 1000 mg l^{-1}. Total BTEX depletion after 10 days and after correction for abiotic losses was 14.7, 67.4, and 67.6%, in the control and in treatments with 100 and 1000 mg cycloheximide l^{-1}, respectively, which confirms the results reported in Table 2.

The sediment extract used for the microcosms was enumerated for protozoa in the presence of 0, 100 and 1000 mg cycloheximide l^{-1}. MPNs calculated after 10 days were 1 and 0.44 cells (g sediment)$^{-1}$ in the presence of 100 and 1000 mg cycloheximide l^{-1}, respectively, while the population was 109 cells (g sediment)$^{-1}$ in its absence. Similar observations were reported when cycloheximide was added to glucose-amended soil, where its addition resulted in a decline in the number of protozoa and a rise in the number of protozoan cysts [46].

3.4. Summary of aerobic inhibitor studies

The use of a eukaryote inhibitor for grazing experiments is predicated on several assumptions [47]. First, the target heterotrophic eukaryotes are inhibited; second, the non-target microorganisms (autotrophic and heterotrophic bacteria) are not inhibited; and third, bacterial growth rates are not stimulated either directly by use of the inhibitor as a substrate or indirectly by the inhibitor lysing cells and thereby increasing bacterial substrate concentrations. The first assumption is accurate as the MPNs in the presence and absence of cycloheximide show that it was an effective protozoan inhibitor. The second assumption is also accurate because cycloheximide did not inhibit bacterial activity at concentrations up to 1000 mg l^{-1} (Table 2). While cycloheximide could have stimulated overall bacterial growth by serving as a carbon source or by lysing cells (assumption 3), it seems unlikely that this would cause a significant increase in the BTEX degradation rate. If BTEX degraders grow on cycloheximide, then the cycloheximide concentration would be expected to have a greater effect on BTEX biodegradation. In fact, the cycloheximide concentration had little effect on the extent of BTEX biodegradation (Table 2). Thus, the enhanced BTEX biodegradation in the presence of a protozoan inhibitor suggests the potential for protozoan grazing to reduce BTEX biodegradation in the tested sediment.

3.5. Anaerobic experiment

Under anaerobic conditions, the two live macrocosms (benzaldehyde only and benzaldehyde+cycloheximide) started utilizing benzaldehyde immediately. Benzaldehyde consumption was similar in these two treatments and after 360 h, total consumption was 62 and 57 mg in the presence and absence of cycloheximide, respectively. Depletion of benzaldehyde in the live macrocosms relative to the abiotic (data not shown), suggested that bacterial populations were utilizing benzaldehyde for growth. Microbial populations (total fermenters, Fe reducers and anaerobic protozoa) were enumerated at various time points during the first 360 h (Fig. 1). The pop-

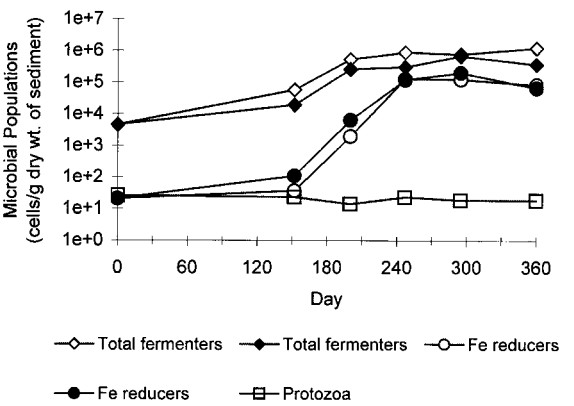

Fig. 1. Microbial populations in benzaldehyde-only and benzaldehyde+protozoan inhibitor treatments. Open symbols represent treatments with benzaldehyde only, closed symbols represent treatments with benzaldehyde plus cycloheximide.

ulations of both total fermenters and Fe reducers were similar after 360 h in the presence and absence of cycloheximide. The increase in Fe reducers is consistent with the $Fe(II)_{aq}$ concentration, which increased in both the presence and absence of cycloheximide from about 55 mg l^{-1} initially to about 260 mg l^{-1} after 360 h. Protozoan populations in the benzaldehyde-only treatment fluctuated around 20 cells per gram of sediment (Fig. 1), while no vegetative protozoa were detected in the benzaldehyde+cycloheximide treatment.

It was hypothesized that bacterial populations (total fermenters and Fe-reducers) would be stimulated by the presence of a protozoan inhibitor but this did not occur. Thus, in the macrocosm system, protozoa did not control the bacterial populations as evidenced by the similarity in benzaldehyde uptake in the presence and absence of cycloheximide. Continuous respiking of benzaldehyde in these two treatments over a period of 1000 h did not change the trend reported for 360 h.

Cycloheximide concentrations in benzaldehyde+cycloheximide and abiotic control treatments decreased during the course of the experiment (data not shown). This may be attributed to adsorption onto the sediment. However, the cycloheximide concentration was over 500 mg l^{-1} after 360 h, and no protozoa were detected in the benzaldehyde+cycloheximide treatment. Thus, the cycloheximide amendment was effective in controlling protozoa. Finally, no inhibition of bacterial populations was observed as evidenced by the similarity in $Fe(II)_{aq}$ production in the presence and absence of cycloheximide.

4. Discussion

The elevated protozoan population in the contaminated region relative to the pristine region at Rocky Point suggested that predation could be a significant regulator of biodegradation in the aquifer. Predator–prey biomass ratios also suggested that protozoa may influence contaminant biodegradation in the aquifer.

Aerobic incubations indicated that the presence of protozoa resulted in decreased biodegradation rates. These data were obtained in an experimental system in which cells were free living rather than attached and this may have enhanced the potential for predation as reported by others [48]. Nevertheless, given previous reports on the presence of protozoa in contaminated aquifers, it seems plausible that protozoan grazing may be a regulator of in situ biodegradation rates. Sinclair et al. [25] reported elevated protozoan populations in a section of an aquifer amended with H_2O_2 relative to an untreated area. Similarly, elevated numbers of protozoa were observed in a sewage-contaminated aquifer [26].

Although anaerobic protozoa were present in the aquifer sediment, protozoa did not adversely affect the rate of anaerobic benzaldehyde biodegradation in the macrocosm system employed here. The absence of an effect may have been due to the rapid degradability of benzaldehyde, toxic effects of benzaldehyde on protozoa, iron fouling or production of biological intermediates that could have inhibited protozoan growth. Because benzaldehyde degraded rapidly, microbial growth may have been sufficiently rapid to negate any adverse effects of grazing. This is consistent with the observations from the aerobic study where cycloheximide was most stimulatory to the slowest degrading BTEX component (*o*-xylene) but had no measurable effect on the biodegradation rates of the most easily degraded components (ethylbenzene and *m*-xylene). The low growth yield of anaerobic protozoa relative to bacteria could explain the absence of a grazing effect. In a study to determine the role of anaerobic ciliates on bacteria in an anaerobic lake system, less than 0.1% of bacterial biomass was consumed per day [49]. Low growth efficiency of anaerobic protozoa was suggested to be the most probable reason.

There are not many studies that have examined benzaldehyde toxicity on protozoa. In one study, the toxicity threshold of benzaldehyde on *Entosiphon sulcatum* (protozoa) was reported to be 0.29 mg l^{-1} based on a cell multiplication inhibition test [50]. However, this organism may have been unusually sensitive to toxicants since 0.5 mg l^{-1} of 2,4-dichlorophenol was also inhibitory [50]. In contrast, there was no evidence of 2,4-dichlorophenol toxicity to 23 strains of free-living amoebae [51]. In this study, the only anaerobic protozoa observed were amoebae. Protozoan MPNs fluctuated around 20 cells (g sediment)$^{-1}$ for 360 h and did not decrease. These populations grew in the presence of benzaldehyde that

was carried over from the inoculum. Thus, there is no evidence to suggest that benzaldehyde exerted toxicity.

In parallel research, the major products of anaerobic benzaldehyde transformation were benzoate and benzylalcohol [52]. The accumulation of these fermentation endproducts was attributed to fouling of the sediment surface as sedimentary Fe was reduced, causing a shift from Fe-reduction to fermentation as the dominant microbial process. Thus Fe-fouling, rather than protozoa, may have been the factor controlling the benzaldehyde biodegradation rate. Benzoic acid toxicity has been reported at 218 mg l^{-1} [50]. Assuming stoichiometric conversion of benzaldehyde to benzoate, 158 mg l^{-1} of benzoate could have been produced in these incubations at the end of 360 h. No report on benzylalcohol toxicity was found. Finally, sulfate concentrations in the groundwater adjacent to the location of sediment excavation were 10–20 μM. Thus, an accumulation of sulfide could not account for a toxic effect.

The absence of a grazing effect on anaerobic benzaldehyde degradation may not necessarily be extended to anaerobic BTEX degradation. Anaerobic BTEX degraders likely grow very slowly which would make this population especially susceptible to the effects of even limited protozoan grazing. This, plus reports of both aerobic and anaerobic protozoa in a fuel-contaminated aquifer [27] indicate that protozoa may be active under a variety of electron-accepting conditions. Further studies of the importance of protozoan grazing in contaminated aquifers are required to determine whether protozoa are a significant regulator of in situ biodegradation rates. The importance of protozoan grazing may also be related to the physical and chemical characteristics of the aquifer, including redox conditions, soil characteristics, groundwater velocity and contaminant characteristics.

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